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# Influence of the Donor Side of Photosystem II on the Photogeneration of Superoxide Radicals and Chlorophyll a Fluorescence

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Direct EPR evidence of the photo-generation of superoxide radicals ($O_2^{-\cdot}$) was obtained by using spin trapping techniques in spinach photosystem II (PSII) membranes. $O_2^{-\cdot}$ was detected by following the formation of 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) superoxide adducts, DEPMPO-OOH. The significant increase of the EPR signal amplitude of DEPMPO-OOH in $NH_2OH$-, $CaCl_2$- and NaCl-treated PSII membranes showed that the oxygen-evolving system has a close relation to the $O_2^{-\cdot}$ production. PSII membranes with inactivated donor side could not prevent the $O_2^{-\cdot}$ production efficiently. Treatments on PSII donor side also influence the maximum level and the kinetics of Chlorophyll (Chl) a fluorescence. Results suggested that manganese cluster and extrinsic proteins might affect Chl a fluorescence in ways different from that happens at the acceptor side of PSII.

**Key words**: photosystem II, donor side, extrinsic protein, manganese, EPR, superoxide, Chl a fluorescence

## INTRODUCTION

Photosystem II is a large membrane-bound multi-protein complex that photochemically catalyzes the oxidation of water and the reduction of plastoquinone. Two chlorophylls are assembled as the reaction center (P680), and the primary electron acceptor is pheophytin (Pheo). At the acceptor side, $Q_A$ and $Q_B$, the one-electron and the two-electron acceptor plastoquinone respectively, accept electrons from $Pheo^-$ in turn, and then transfer them to PQ pool in thylakoid membranes. While at the donor side of P680, water molecules are split into $H^+$ and $O_2$ by a sequence of four electron transfer steps of the manganese cluster, which transfers electrons to tyrosine 161 of D1 protein (TyrZ), then to $P680^+$. Three extrinsic proteins, with apparent molecular masses of 33, 23 and 17 kDa, are located at the lumen side of PSII complex and required for oxygen evolution.

The photo-generation of $O_2^{-\cdot}$ in PSII was firstly indirectly studied by means of ferricytochrome c reduction and voltammetric detection [1,2]. Until recently, straightforward EPR evidence was obtained for $O_2^{-\cdot}$ production in wheat PSII membranes with a novel spin trap DEPMPO [3]. In the present study, this spin trapping EPR probe was taken to study the correlation of $O_2^{-\cdot}$ formation with oxygen-evolving complex. Chl a fluorescence are the most widely used technique in photosynthesis research. We also use different treated PSII membranes to study the relationship between the oxygen-evolving complex and Chl a fluorescence.

## MATERIALS AND METHODS

PSII membranes were prepared from market spinach leaves following the method of Berthold et al. [4] with slight modification. Freshly isolated PSII membranes were suspended in SMN solution (0.4 M sucrose, 10 mM $MgCl_2$, 15 mM NaCl, 50 mM MES-NaOH, pH 6.5) and stored at 77 K until use.

For NaCl treatment, PSII membranes (1 mg Chl/ml), diluted with equal volume of 3 M NaCl, were kept in room light at 4°C for one hour. Membranes were then collected after the centrifugation of 40,000×g for 20 min and washed once with SMN. For treatments with $CaCl_2$, NaCl/urea and $NH_2OH$, PSII membranes (1 mg Chl/ml) were diluted with an equal volume of 2 M $CaCl_2$, 2.6 M urea/0.2 M NaCl, or 10 mM $NH_2OH$. Treatments were carried out at 4°C in darkness for 30min. Others were similar to that for NaCl treatment. Resultant pellets of

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PSII membranes were suspended in SCN solution (0.4 M sucrose, 10 mM $CaCl_2$ and 180 mM NaCl, 50 mM Mes-NaOH, pH 6.5).

For EPR measurement, PSII membrane samples were suspended in 10 mM sodium phosphate buffer (pH 6.5) containing 100 mM sucrose, 5 mM $MgCl_2$, 50 mM NaCl. Illumination with light intensity of 2000μE $m^{-2}$ $s^{-1}$ was directly given to samples (150 μg chl /ml) in the EPR cavity with a 500 W Xenon lamp at room temperature. EPR cavity was protected with a water filter from far-red irradiation. A long-pass filter was used to cut off light less than 400 nm. EPR spectra at X-band were recorded at room temperature by a Varian E-112 spectrometer.

Chl a fluorescence induction curves were measured with a lab-assembled fluorometer at 20°C. Illumination was provides by arrays of twenty blue light emitting diodes (LIAG0513). Sample were dark adapted for 20 min before illumination. Chl a fluorescence inductions were measured with a photodiode (SL254), protected with a red filter. The chlorophyll concentration of samples was 10μg/ml.

## RESULTS AND DISCUSSION

There was no EPR signal detected in darkness for PSII membranes in the presence of 10 mM DEPMPO (data not shown), indicating no $O_2^{-\cdot}$ production. PSII membranes were then illuminated with 2000 μE $m^{-2}$ $s^{-1}$ in the presence of 10 mM DEPMPO. Marked EPR spectrum of DEPMPO-OOH (Fig.1a) was observed, well consistent with previous observation [5].

In order to study the influence of structural change at the donor side of PSII on $O_2^{-\cdot}$ production, PSII membranes were treated with NaCl, $CaCl_2$ and $NH_2OH$ respectively. 17 and 23 kD extrinsic proteins were depleted in NaCl-treated PSII membranes. $CaCl_2$ (and NaCl/urea) treatment liberated all extrinsic proteins from PSII membranes. $NH_2OH$-treated PSII membranes were devoid of manganese cluster of PSII, but was still containing three extrinsic proteins. As to $NH_2OH$-, $CaCl_2$- and NaCl-treated PSII membranes, DEPMPO-OOH adducts were also observed after illumination of 5 min, as shown in Fig.1b, c, d, respectively. Judging from the height of the fourth-peak of DEPMPO-OOH spectra, relative amounts of superoxide radicals, showed following order for different membranes: $CaCl_2$-treated > $NH_2OH$-treated > NaCl-treated > control. Enhancement on $O_2^{-\cdot}$ generation was clearly shown for all treated PSII membranes. EPR signal of DEPMPO-OOH was totally abolished by adding 10 μM DCMU

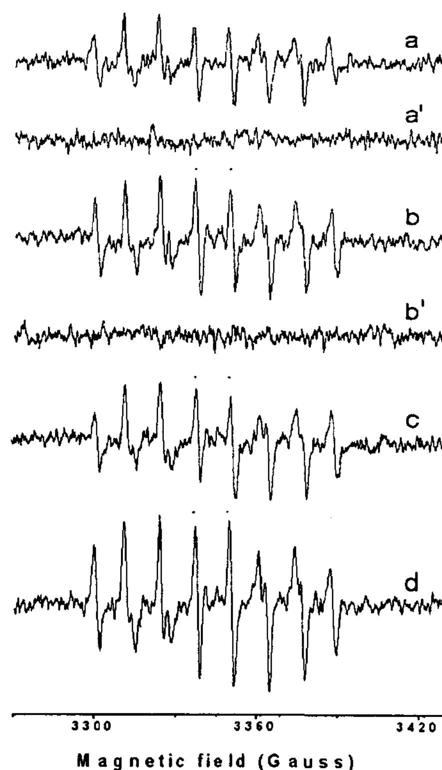


Figure 1. EPR spectroscopy obtained from: a, Control; a', a + 10 μM DCMU; b, $NH_2OH$-treated; b', b + 10 μM DCMU; c, NaCl-treated; d, $CaCl_2$-treated PSII membranes, after 5 min illumination (λ>400 nm) in suspension containing 150 μg chl /ml sample, 10 mM DEPMPO, 10 mM sodium phosphate (pH 6.5), 100 mM sucrose, 5 mM $MgCl_2$ and 50mM NaCl. Circle o shows the characteristic lines of the $O_2^{-\cdot}$ adducts. Spectrometer parameters were set as follows: microwave frequency, 9.17 GHz; modulation frequency, 100 KHz; microwave power, 50 mW; modulation amplitude, 0.5 G; time constant, 0.128 s; receiver gain, $5\times10^4$.

into PSII membranes (Fig.1a'). For $NH_2OH$-treated samples, adding 10 μM DCMU also suppressed the formation of $O_2^{-\cdot}$ (Fig.1b'). Above results suggest that a direct contribution of water oxidation to $O_2^{-\cdot}$ formation is unlike, but PSII membranes with inactivated donor side could not prevent the $O_2^{-\cdot}$ production efficiently, implying that extrinsic proteins and Mn at the donor side are very important to eliminate $O_2^{-\cdot}$. Since the chlorine concentration (50mM) in sodium phosphate buffer is not high enough to stabilize the Mn cluster when all three extrinsic proteins were depleted, part of Mn cluster in $CaCl_2$-treated PSII membranes might be liberated during EPR measurement. The release of

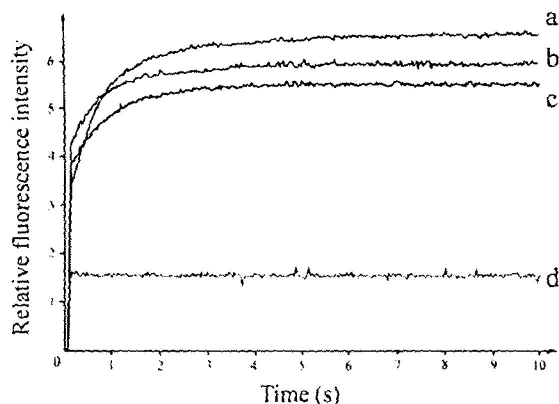

Figure 2. Chl a fluorescence dynamics of control and treated washes PSII membranes. a, control; b, NaCl-treated; c, NaCl/urea-treated; d, $NH_2OH$-treated. The chlorophyll concentration of sample was 10μg/ml.

extrinsic proteins and manganese might produce additive influence on $O_2^{\cdot-}$ formation, and caused more $O_2^{\cdot-}$ generation.

Figure 2 shows Chl a fluorescence dynamics of control and different treated PSII membranes. It can be easily seen that the maximum fluorescence (Fm) of all treated PSII membranes are lower than that of control. Compared to other two treatments, $NH_2OH$ treatment caused more significant decreasing in Fm. More over, the fluorescence dynamics of $NH_2OH$-treated samples was totally different with others. Results suggest that the donor side of PSII causes influence on Chl a fluorescence induction, and manganese cluster and extrinsic proteins might effect in different ways.

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